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KvLQT1 and *KCNE1* K+ Channel Gene Polymorphisms in Long QT Syndrome

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Authors' contributions

This work was carried out in collaboration between all authors. Author SFQ has carried out the molecular analysis described in this manuscript and has compiled the manuscript. Author AA has helped to carry out the in-silico analysis described in this manuscript. Author APJ has managed the literature survey. Author AV has interpreted the results described in this manuscript. The probands described in this manuscript have been diagnosed for LQT syndrome by authors MPJ, CN and JS at their respective hospitals. Author KT has carried out the sequencing analysis of the samples described in this manuscript. As the corresponding author, the concept, design and compilation of this manuscript has been carried out by author PN. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Long QT Syndrome (LQTS), a disorder of the cardiac repolarization process with prolongation of the QT interval (QTc ≥0.46 seconds), is an ion-channelopathy. Mutations

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in either *KCNQ1* or *KCNE1* genes are susceptible to LQTS. Hence, screening of *KCNQ1* and *KCNE1* genes is taken up to evaluate the genetic correlation of these genes in Long QT patients of Indian origin.
A total of 33 Long QT Syndrome patients and 100 healthy subjects were enrolled for the present study. PCR-SSCP protocol was utilised for screening of *KCNQ1* and *KCNE1* genes followed by In-silico and statistical analysis.
The clinical profile of the Long QT syndrome patients in our study revealed a higher percentage of females with the mean age also being higher in females when compared to males. The two variations (S546S and IVS13+36A>G) in *KCNQ1* and the S38G polymorphism in *KCNE1* gene were identified and their association with Long QT syndrome is being reported for the first time in Indian population. S546S is located in the *KCNQ1* C terminus close to this domain and IVS13+36A>G is located in the intronic region in close proximity to the coding region for C-terminal domain; these may therefore affect the functional protein through non-assembly. S38G leads to a substitution of serine to glycine at 38th amino acid position (S38G) in the transmembrane domain of KCNE1.
Our study reports compound heterozygosity/genetic compound ofS546S and IVS13+36A>G of *KCNQ1* gene. Haplotype frequencies and linkage disequilibrium analysis revealed a significant association between the three biomarkers. Compound heterozygosity of the polymorphisms influence downstream signalling and KCNQ1-KCNE1 interactions.

Keywords: Long QT syndrome; compound heterozygotes; KCNQ1; KCNE1; linkage disequilibrium; C-terminal domain.

1. INTRODUCTION

Long QT Syndrome (LQTS), a congenital disorder, characterized by prolongation of the QT interval (QTc ≥0.46 seconds) affects 1 in 7,000 individuals of the general population.LQTS is associated with recurrent syncope due to a transient, rapid, polymorphic ventricular tachycardia, also known as torsade de pointes, and sudden death. Genetic investigations demonstrated LQTS susceptibility genes to be of cardiac ion channels and/or regulatory proteins involved in localization of sodium or calcium channels to the sarcolemma, hence, long QT syndrome is a disease of ion channel dysfunction [1].

The voltage-gated potassium channel, KQT-like subfamily, member 1 (KCNQ1), encodes pore-forming voltage gated K+ channel subunit KvLQT1 and is widely expressed in myocardial tissue. Hence it plays a key role in repolarization of the cardiac action potential and transport of water and salt in epithelial tissues. [2] Each KCNQ1 subunit has six transmembrane segments (S1–S6), with S1–S4 segments serving as a voltage-sensor domain, S5–S6 segments forming a pore domain and four KCNQ1 subunits forming the ion channel [3]. KCNQ1 channels only form homotetramers and function in non-excitable as well as excitable tissues. KCNQ1 channels co-assemble with KCNE1 peptides, affording a complex with different gating properties and pharmacological sensitivities. The KCNQ1-KCNE1 complex generates cardiac IKs current in the heart and provides an avenue for K+ to enter the endolymph in inner ear [4]. The biophysical properties of the KCNQ1 current are dramatically altered when its subunit associates with KCNE1.Mutations in KCNQ1 or KCNE1 maybe fatal for life-threatening cardiac arrhythmias [5,6,7,8,9].

KCNQ1 gene is localised to 11p15.5, and mutations in the *KCNQ1* gene result in type-1 long-QT syndrome. Analysis of the gene demonstrated a truncated isoform, which encodes

a 549-amino acid protein [10]. KCNE1 is a 129-amino acid minK protein containing one transmembrane-spanning domain. It is encoded by *KCNE1* gene located at 21q22.1-22.2 which is implicated in LQT type-5 [11,12]. Mutations in the *KCNQ*1 gene account for the vast majority of congenital LQTS with most missense mutations being located in the conserved core domain of the protein and in the cytoplasmic C-terminal domain. Functional expression studies have shown that mutations within the core domain of the KvLQT1 protein seem to cause loss of function and dominant-negative effect. A P-domain mutation with a very mild functional effect has also been reported. One C-terminal mutation, R555C, has been associated with a 'formefruste' of LQTS that leads to a functional channel with an altered voltage-dependence [13].

Mutations in either *KCNQ1* or *KCNE1* decrease the conductance of the complex and prolong the cardiac action potential, leaving individuals with these mutant proteins susceptible to long QT syndrome. Hence, this study focuses on screening *KCNQ1* and *KCNE1* genes in Indian population to understand the genetic correlation of these genes in patients with Long QT syndrome. We report two variations in *KCNQ1* and a polymorphism in *KCNE1* genes and their pathogenic potential was examined by In-silico analysis.

2. METHODOLOGY

2.1 Study Subjects

Blood samples were collected for molecular analyses from confirmed 33 LQTS probands and their available family members from Care Hospitals, Hyderabad, Sri Jayadeva Institute of Cardiovascular Science and Research, Bangalore and Institute of Maternal and Child Health,Calicut Medical College, Calicut. This study has been approved by the Institutional Ethics Committee, Dept. of Genetics, Osmania University, Hyderabad and informed written consent was obtained from the probands and their family members. Blood samples from 100 controls (50 M: 50 F), without any history of cardiovascular or systemic conditions, were collected from Osmania General Hospital, Hyderabad for comparative analysis.

2.2 Molecular Analyses

Genomic DNA was isolated from peripheral blood samples by standard protocols in 100 controls, 33 probands and their family members. The DNA sequences corresponding to *KCNQ1* and *KCNE1* genes were amplified using the primer sets as described by Syrris et al [14]. Fragments were amplified on Eppendorf Thermal cycler Gradient in the presence of 1 U Taq DNA polymerase, 0.2 mMdeoxyribonucleotide, 1.5 mM $MgCl_2$, 100 ng forward and reverse primers and genomic DNA. The PCR products were subsequently screened by Single Stranded Conformational polymorphism (SSCP) according to standard procedures and the gels were visualized by silver-staining. The variations observed, were confirmed by commercial sequencing (HiSeq 2000 sequencing machine, Illumina, CA).

2.3 Statistical Analysis

Fisher's exact test and odds risk estimate were computed for possible genotype association. Interactive SNPs were analyzed by means of logistic regression (OR) to determine the significance of risk genotypes at 95% confidence interval (CI) followed by haplotype frequency computation by the EM algorithm using SNPstat software [15]. Linkage disequilibrium was inferred by Haploview software [16].

2.4 In-Silico Analysis

In-silico analysis was also carried out to elucidate the mRNA secondary structure changes (GENEBEE software), splice site changes (www.cbs.dtu.dk/services/NetGene2/), possible binding site variations for SnRNP's involved in spliceosome formation (http://sfmap.technion.ac.il/) and possible changes in protein primary structure (http://asia.ensembl.org) that resulted due to the observed exonic variations.

3. RESULTS

3.1 Clinical Profile

Data presented in Table 1 indicates mean age of 18.2 ±15.7yrs in males and females at 22 ±16.6 yrs in LQTS patients. 36.36% of the males exhibited Long QT syndrome when compared to 63.63% of females. The mean age at onset in all the Long QT syndrome patients was found to be 17.6 ±17.4yrs. 27.27% of the patients exhibited consanguinity and history of sudden deaths in their family. Clinical profile indicated that 63.63% of the patients had an episode of syncope and 9.09% were deaf. Mean QTc interval was found to be 493 ± 45.8 msec in males and 476 ± 51.7 msec in females. Patients with acquired Long QT Syndrome (aLQTS), exhibited hypokalemia with the serum potassium levels as low as 3.3 mmol/L. 84.84% of our patients were non-vegetarian.

Table 1. Comparison of epidemiological variables in controls and LQTS patients

Variable	Controls n(%)	LQTS patients n(%)
aLQTS	-	aLQTS – 6 (18.18%)
cLQTS		cLQTS - 27 (81.81%)
Gender	Females-21 (63.63%)	Females-29.4 **±** 10.6
	Males-12 (36.36%)	Males-30.4 **±** 8.07
Consanguinity	-	9 (27.27%)
Nutrition	Non-veg-89 (89%)	Non-veg-28 (84.84%)
	Veg-11 (11%)	Veg-5 (15.15%)
Syncope	-	21 (63.63%)
Deafness	-	3 (9.09%)
F/h Sudden deaths	-	9 (27.27%)
Variable	**Controls (Mean+SD)**	**LQTS patients (Mean+SD)**
QTc in msec	Females- 410 Males-390	Females-476 ± 51.7
		Males-493 ± 45.8
Age in yrs	Females-29.4 **±** 10.6	Females-22 **±** 16.6
	Males-30.4 **±** 8.07	Males-18.2 **±** 15.7
Age at onset in yrs	-	17.6 **±** 17.4
Serum potassium (mmol/L	3.4-5.5	3.3

3.2 Molecular Results

3.2.1 KCNQ1

PCR-SSCP analysis was carried out for all the 16 exons of *KCNQ1* gene revealed variations in exons 3, 4 and 13. Variations observed in exon 3 and 4 of *KCNQ1* in a proband (P6) and

his family members highlighting the hot spot of these exons [Qureshi et al, personal communication].

PCR-SSCP analysis of exon 13 of *KCNQ1* revealed 3 types of band patterns in both controls and LQTS patients (Fig. 1).

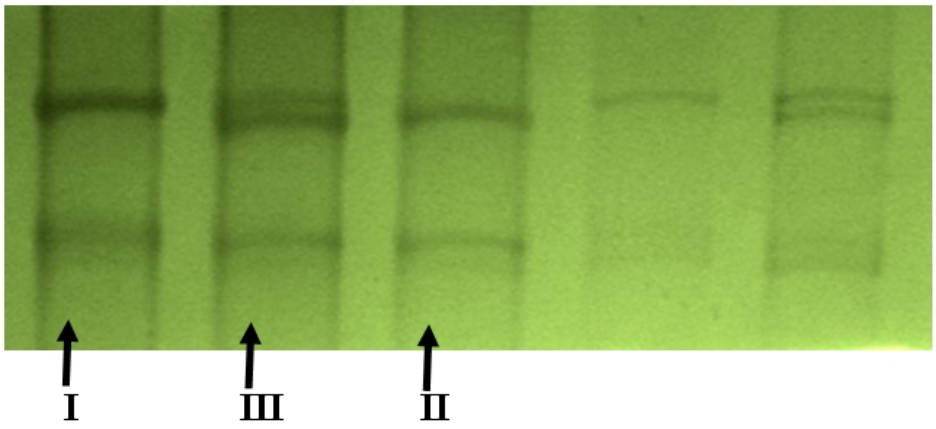
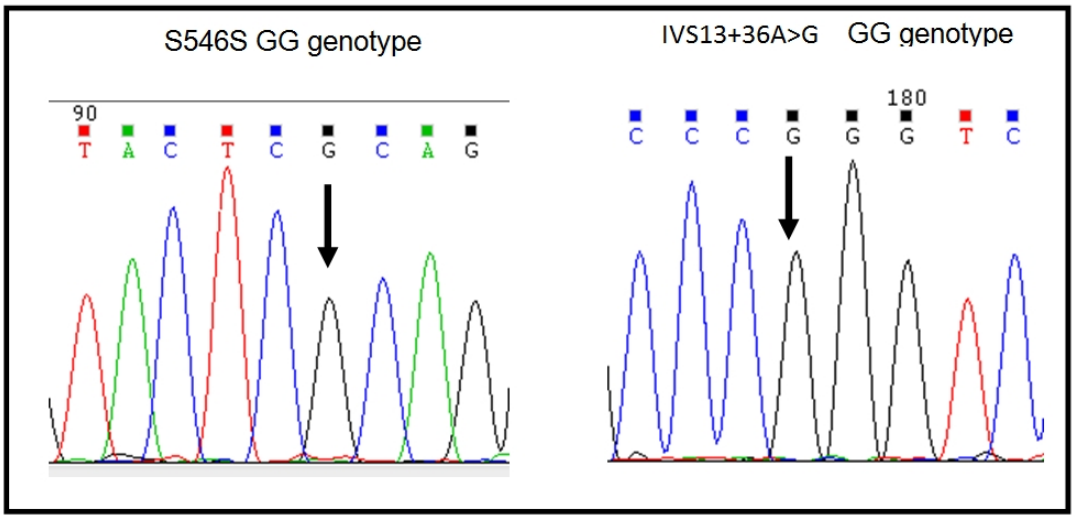


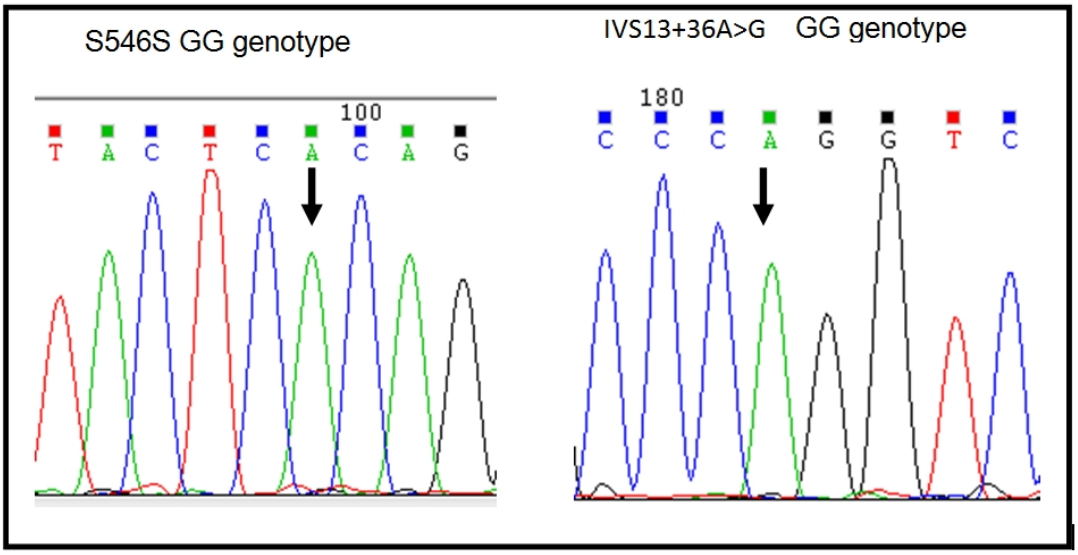
Fig. 1. SSCP pattern of Exon-13 of *KCNQ1*

Samples for each band pattern on commercial sequencing revealed 2 SNPs:

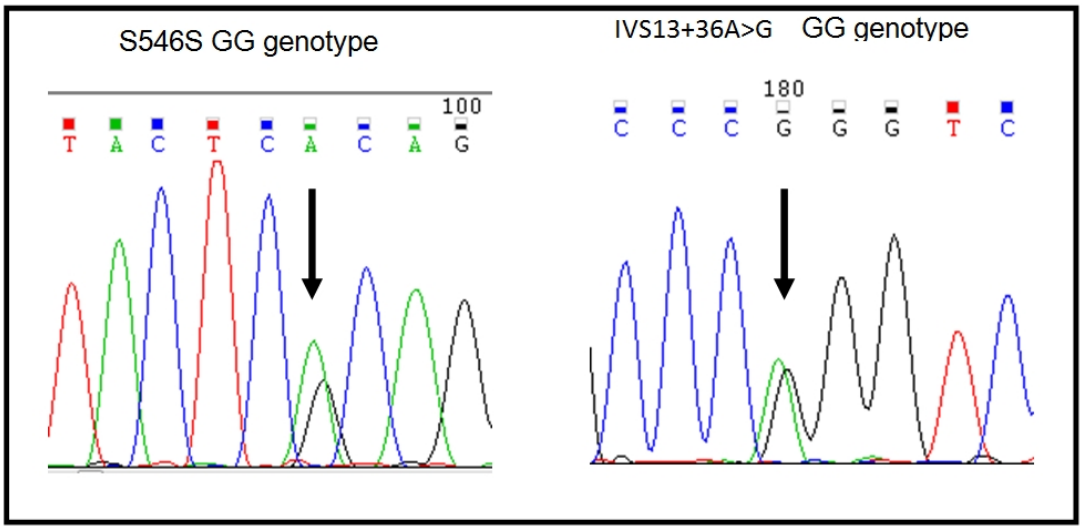
- G > A transition at 2737237 in Exon 13 leading to a neutral substitution S546S – rs1057128 (Fig. 2) and
- A>G transition at 2737320 in Intron 14 -IVS13+36A>G(Fig. 2).



a) Electropherogram of Band pattern 'I' showing GG genotype of nt2737237 (S546S) and IVS13+36A>G.



b) Electropherogram of Band pattern 'II' showing AA genotype of nt2737237 (S546S) and IVS13+36A>G



c) Electropherogram of Band pattern 'III' showing AG genotype of nt2737237 (S546S) and IVS13+36A>G

Fig. 2.Electropherograms indicating the transition of G > A at 2737237 (S546) and A>G at 2737320 (IVS13+36A>G)of *KCNQ1* gene

Table 2 clearly indicates that an individual does not carry variant allele of both the polymorphisms, instead each individual is found to carry wild type allele of one SNP and variant allele of the other leading to compound heterozygosity/Genetic compound.

Table 2. Genotypes for S546S and IVS13+36A>G SNPs in controls and LQTS patients

Gene	exon	SNP	Genotype of band pattern 'I'	Genotype of band pattern 'II'	Genotype of band pattern 'III'
KCNQ1	Exon 13	G > A nt2737237 (S546S)	GG (wild type)	AA (variant)	AG (heterozygote)
KCNQ1	Intron 14	IVS13+36A>G	GG (variant)	AA (wild type)	AG (heterozygote)

3.2.2 KCNE1 gene

KCNE1 gene has a single exon which is split into two fragments for analysis. The PCR-SSCP analysis of the second fragment did not reveal any variation but the first fragment of *KCNE1* revealed a variation with three types of band patterns I, II, III (Fig. 3).

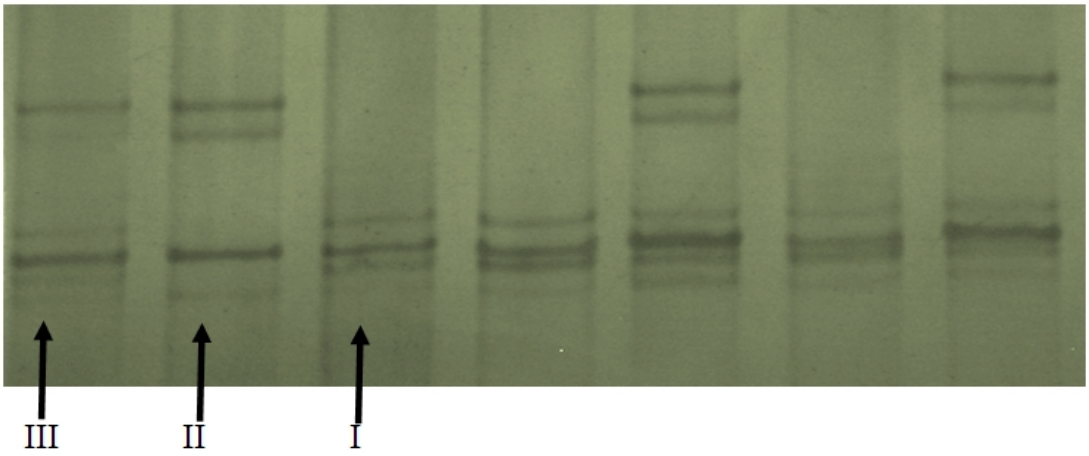


Fig. 3. SSCP pattern of 1st fragment of *KCNE1*

Commercial sequencing of samples for each band pattern revealed a transition A>G at 542position in *KCNE1* single exon leading to a substitution of serine to glycine at 38th position S38G- rs17846179 (Fig. 4).

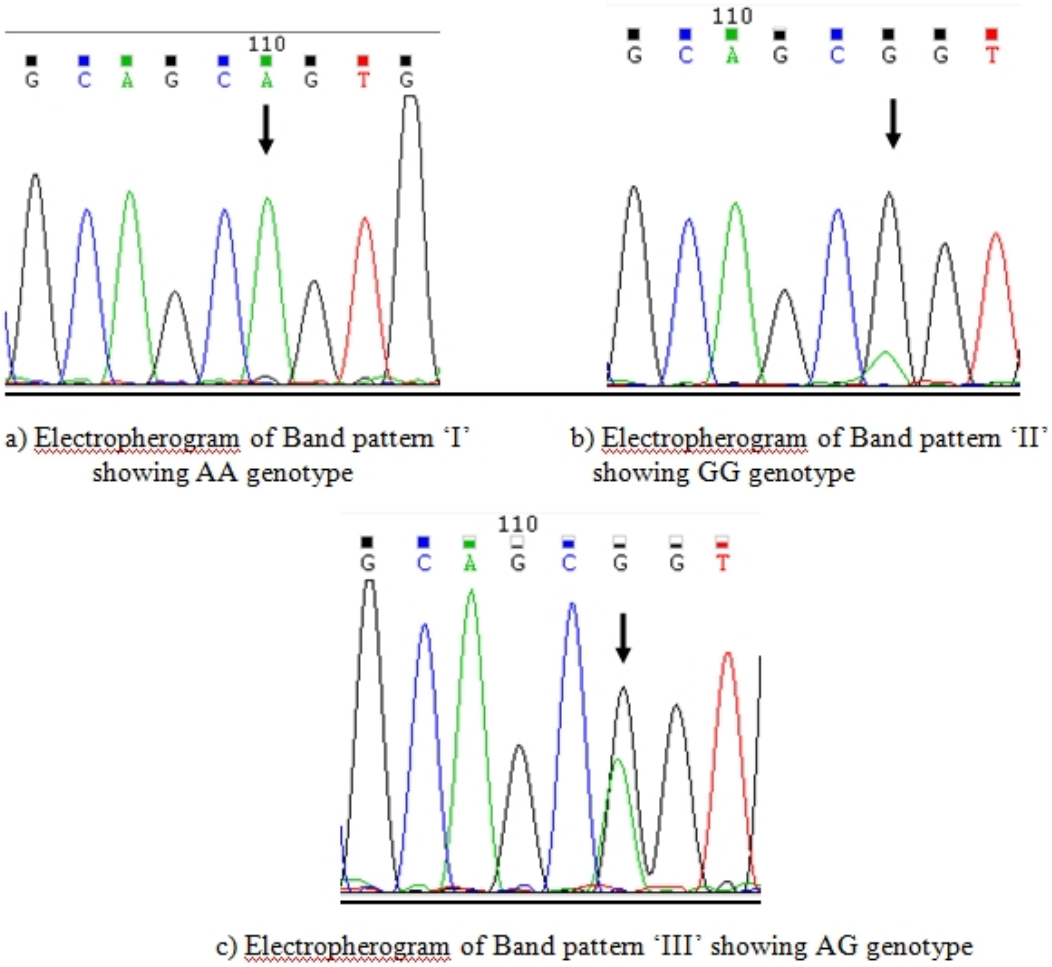


Fig. 4. Electropherograms indicating the transition of 542 A>G (S38G) in *KCNE1* gene

3.3 Statistical Analysis

For the S546S and IVS13+36A>GSNPs, the genotypic frequencies of AA, AG and GG were found to be 5%, 55% and 40% in controls and 16%, 28% and 56% in LQTS patients respectively. A higher frequency of the variant 'AA' (16%) genotype in LQTS patients was observed when compared to controls (5%) indicating the preponderance of 'A' allele as a risk allele. However, no significant difference was observed in the allelic frequency distribution, indicating the gene pool to be in equilibrium (Table3). Odds risk estimates were calculated using SNPSTAT software which generated four models. The odds ratio was found to be 0.36folds higher for 'AG' when compared to GG and AA genotypes (95% CI 0.15-0.89; p = 0.014) (Table4).

The genotypic frequencies for the A>Gtransition at 542 position (S38G) were 4%, 55% and 41% in controls and 12%, 38% and 50% in LQTS patients respectively for AA, AG and GG genotypes. Allelic frequencies were similar in LQTS patients and controls with the

distribution being in equilibrium (Table3). Odds risk estimates of the genotypes did not reveal any significant variation (Table4).

Table 3. Genotypic and allelic frequency distribution of the 3 polymorphisms in controls and LQTS patients of *KCNQ1*

SNP	Genotype	Controls n (%)	LQTS patients n (%)	Allele	Controls	LQTS patients
S546S	AA	5 (5)	5 (16)	G	0.68	0.7
(rs1057128) &	AG	**55 (55)**	**9 (28)**	A	0.32	0.3
IVS13+36G>A	GG	40 (40)	18 (56)			
S38G	AA	4 (4)	4 (12)	G	0.68	0.69
(rs17846179)	AG	**55 (55)**	**12 (38)**	A	0.32	0.31
	GG	41 (41)	16 (50)			

Table 4.Odds risk estimates of genotypes and alleles in LQTS patients compared to control group for the 3 polymorphisms identified

SNP	Model	Genotype	OR (95% CI)	p value
S546S	Codominant	GG AG	1.00	
(rs1057128)		AA	**0.36 (0.15-0.89)**	
			2.22 (0.57-8.65)	**0.014**
&	Dominant	GG	1.00	0.11
		AG/AA	0.52 (0.23-1.16)	
IVS13+36A>G	Recessive	GG/AG	1.00	0.066
		AA	3.52 (0.95-13.06)	
	Overdominant	GG/AA	1.00	**0.0072**
		AG	**0.32 (0.13-0.76)**	
	G vs A		0.877(0.454-1.685)	0.79
S38G	Codominant	GG	1.00	0.11
(rs 17846179)		AG	0.56 (0.24-1.31)	
		AA	2.56 (0.57-11.50)	
	Dominant	GG	1.00	0.37
		AG/AA	0.69 (0.31-1.55)	
	Recessive	GG/AG	1.00	0.1
		AA	3.43 (0.81-14.59)	
	Overdominant	GG/AA	1.00	0.084
		AG	0.49 (0.22-1.11)	
	G vs A		0.988(0.515-1.889)	1.00
				$p \leq 0.05$

3.4 Haplotype Frequencies & Linkage Disequilibrium (LD) Analysis

Haplotype frequencies for the various allelic combinations between the three polymorphisms were computed for their possible association with LQTS (Table 5).Frequency of GGGhaplotype was highest in controls (0.675) and LQTS patients (0.6385). Interestingly, the GGA haplotype was present only in LQTS patients (0.0646). There was a significant difference in the GGA haplotype frequency in patient group ($p<0.0001$) when compared to

controls. The increased significance of the association of the GGA haplotype with Long QT syndrome indicates a functional role of the compound haplotypes in disease susceptibility.

Table 5. Haplotype frequencies of the 3polymorphisms in controls and LQTS patients

Haplotype	Haplotype frequency		p
	Controls	LQTS patients	
GGG	0.675	0.6385	-
AAA	0.315	0.2479	0.8
AAG	0.01	0.049	0.14
GGA	**NA**	**0.0646**	**<0.0001**
			p≤0.05

A pair-wise comparison of the three polymorphisms, depicting the LD measures is represented in Fig. 5. Significant D' values were observed for the S546S &IVS13+36A>GSNPs (D'=0.99), S546S and S38G (D'=0.92) and IVS13+36A>G and S38G (D'=0.92),indicating a strong/tight linkage disequilibrium between the SNPs.

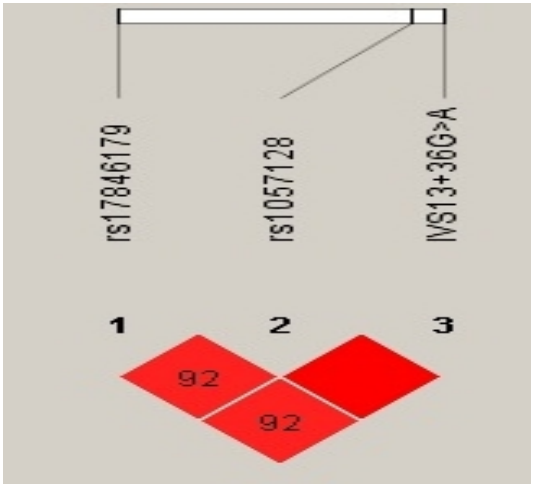


Fig. 5.Pairwise Linkage disequilibrium analysis of *KCNQ1* and *KCNE1* polymorphisms

3.5 In-silicoAnalysis

In case of S546S polymorphism, mRNA secondary structure prediction indicated that the variation lead to a change in the secondary structure of mRNA with an increase in free energy of the variant (-69.3 kkal/mol) when compared to the wild type (-73.7 kkal/mol). Due to the variant allele, splicing factor binding sites were also found to vary for SRp40, NOVA1 with the formation of a new binding site for QK1 motif. These variations might alter the downstream signalling, thus affecting the final stoichiometry of the protein. The 2737237G > A transition in exon 13 leads to a neutral substitution of serine at 546th amino acid (S546S-rs1057128) [17] encoding the C-terminus of KCNQ1 [10]. An increase in usage of codon UCA (0.15) with a variant A allele when compared to codon UCG (0.05) was observed.

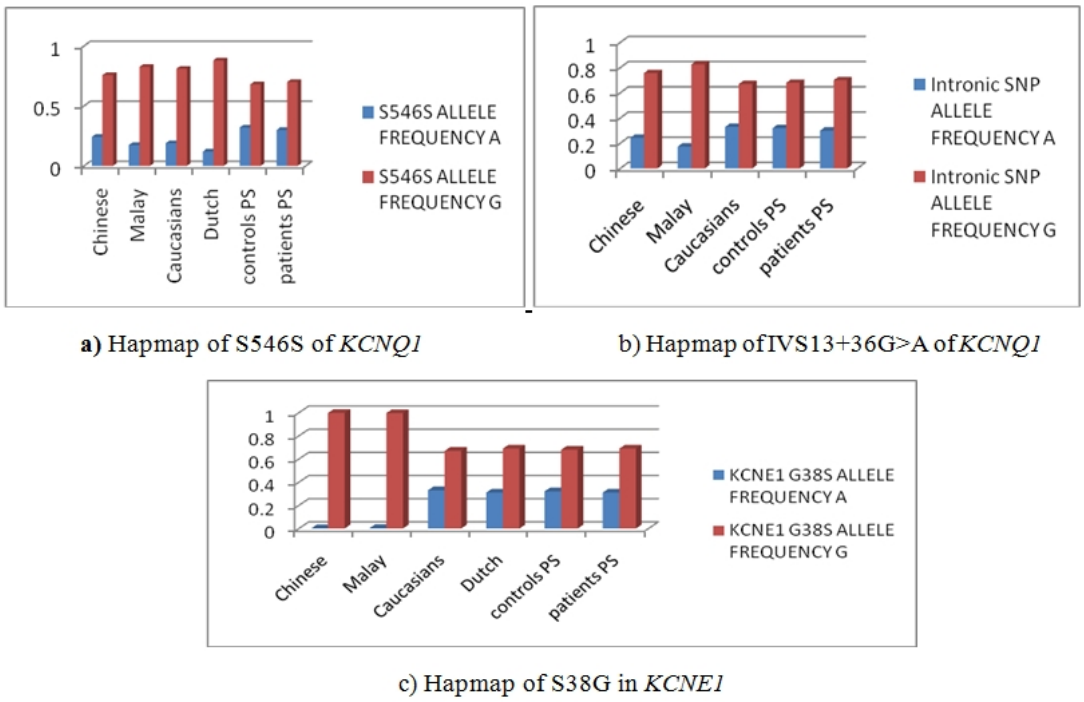
mRNA secondary structure prediction indicated that the intronic variation IVS13+36A>G lead to a loss of stem-4 and formation of an aberrant stem-3 with a decrease in free energy of the

variant (-80.0 kkal/mol) when compared to the wild type (-73.7 kkal/mol). The IVS13+36G>A SNP variant allele caused variations in the splicing factor binding sites for SF2ASF, SC35 and hnRNPH/F motifs.

In case of S38G variant of *KCNE1* gene, secondary mRNA structure prediction indicated that the variation lead to a change in the free energy of stem 4 of secondary structure of mRNA which in turn increased the free energy of the variant (-43.2 kkal/mol) when compared to the wild type (-43.9 kkal/mol). The 542 A>G transition resulted in substitution of serine to glycine at 38th amino acid position (S38G) wherein a polar, hydrophilic, essential serine is being replaced by a non-polar, hydrophobic, non-essential glycine, as explained by Dayhoff's mutation odds matrix [18,19]. The PAM250 scoring matrix gives S38G a score of +1 indicating that the amino acid replacement occurs 1.25 times as frequently as expected by chance [20].

Splice site prediction did not reveal any variation in the splice sites due to the three polymorphisms.

Hapmap was created for all the three polymorphisms to compare the allelic frequencies of Indian population with other populations, which did not reveal any variation in the allelic frequency distribution with respect to S546S and IVS13+36A>G SNPs (Figs. 6a & 6b). However, complete lack of A allele of S38G in the Chinese and Malay populations was observed, emphasizing the genetic diversity in Indian population (Fig. 6c).



a) Hapmap of S546S of *KCNQ1*

b) Hapmap of IVS13+36G>A of *KCNQ1*

c) Hapmap of S38G in *KCNE1*

Fig. 6. Hapmap of all the 3 polymorphisms

4. DISCUSSION

The IKs channel is formed by co-assembly of KCNQ1 and KCNE1. The biophysical properties of the KCNQ1 current are dramatically altered when its subunit associates with KCNE1. In the presence of KCNE1, there is an increase in current amplitude, slow onset of activation, depolarizing shift in the voltage dependence of activation, increase in single channel conductance, removal of inactivation and control of deactivation kinetics by C-terminus. Mutations in KCNQ1 or KCNE1 genes can act as biomarkers for life-threatening cardiac arrhythmias such as long QT (LQT) or short QT syndromes [5,6,7,8,9]. S546S (rs1057128) which is located in the KCNQ1 C-terminus close to assembly domain may affect the functional protein [2]. The loss-of function effects of the KCNE1 S38G allele was reported, to have the potential to predispose to early afterdepolarizations [21].

Our study revealed a higher female preponderance with the mean age being higher in females when compared to males. But, the mean QTc interval was found to be higher in males (493 ± 45.8) than female (476 ± 51.7) patients supporting the less affected sex to be more prone to severity of the disease. Studies have reported the association of synonymous variant rs1057128 (S546S) and nonsynonymous variant rs12720449 (P448R) of *KCNQ1* with Long-QT syndrome in European and Chinese individuals [2]. The association of the rare allele of IVS2-128 A and rs727957 T of *KCNE1* gene associated with the prolongation of the QTc length in healthy Caucasian subjects was also reported [9]. The two variations (S546S and IVS13+36A>G) in *KCNQ1* and the S38G polymorphism in *KCNE1* gene identified in this study is the first to report polymorphisms and their association with LQTS in the Indian population.

The ‘AG’ genotypic frequency was found to be higher in controls when compared to LQTS patients, with respect to all the three polymorphisms. The observations revealed a significant association of ‘AG’ genotype of S546 and IVS13+36A>G with LQTS polymorphisms (OR=0.36; 95%CI 0.15-0.89; p=0.014). Our study is in concordance with Chen et al [2] who described the minor allele of S546 (rs1057128) to be significantly associated with decreased TG (triglyceride) levels in Han subjects. Studies have reported *KCNE1* S38G polymorphism in healthy subjects in two different populations [21, 9]. The haplotype analysis indicated a significant association of the ‘GGA’ haplotype ($p<0.0001$) with LQTS and a strong linkage disequilibrium between the three polymorphisms based on LD analysis.

In-silico analysis of S546S polymorphism of *KCNQ1* gene revealed an increase in free energy of variant mRNA secondary structure which may lower its thermodynamic stability. Splicing factor binding sites were found to vary for SRp40, NOVA1 with the formation of a new binding site for QK1 motif. The variations predicted in mRNA secondary structure and splicing factor binding sites may affect gene regulation with possible variations in protein structure and function due to bias in codon usage [2]. As per previous studies, a small domain between residues 589 and 620 in the KCNQ1 C-terminus may function as an assembly domain for KCNQ1 subunits. In the absence of this domain, KCNQ1 C-termini fail to assemble, and functional potassium channels are not produced [2]. Since, S546S is located in the KCNQ1 C-terminus close to this domain, it may affect the functional protein through non-assembly. Our results indicate that this synonymous variation can also affect the thermodynamic stability of mRNA secondary structures and splicing factor binding sites, therefore, synonymous mutations may not be neutral in evolution but may have a critical role in protein folding and chaperoning.

The intronic variation IVS13+36A>G of *KCNQ1* gene reported by Koo et al [17] in healthy subjects leads to a loss of stem-4 and the formation of an aberrant stem-3 in the variant as indicated by mRNA secondary structure prediction. The IVS13+36A>G SNP variant allele caused variations in the splicing factor binding sites for SF2ASF, SC35 and hnRNPH/F motifs. Since, this polymorphism is located in the intronic region in close proximity to the coding region for C-terminal domain; the mRNA secondary structure and splicing factor binding site predicted variations may influence the C-termini of the protein.

mRNA secondary structure prediction of S38G SNP of *KCNE1* gene indicated that the variation lead to an increase in the free energy of stem 4 of secondary structure of mRNA thus decreasing the thermodynamic stability. This polymorphism leads to a substitution of serine by glycine at 38th amino acid position (S38G) in the transmembrane domain of *KCNE1.* A polar, hydrophilic, essential serine is replaced by non-polar, hydrophobic, non-essential glycine. It has been shown that the transmembrane region of *KCNE1* binds directly to the pore region of *KCNQ1* to regulate channel activity [3,5,6]. Hence, this substitution may lead to variations in the stoichiometry of *KCNQ1* and *KCNE1* complex finally leading to variations in repolarisation by the potassium channel.

Our study reports compound heterozygosity/genetic compound ofS546S and IVS13+36A>G of *KCNQ1* gene. This phenomenon can lead to the interaction of the two polymorphisms which might further lead to variation in the *KCNQ1* potassium channel. Further functional and large cohort studies are required to dissect the mechanisms of *KCNQ1-KCNE1* gating and may lead to the discovery of a potent activator of Q1/E1 complexes for the treatment of cardiac arrhythmias.

5. CONCLUSION

A significant association of *KCNQ1* gene S546S and IVS13+36A>G polymorphisms with Long QT syndrome in the Indian population is being reported. These polymorphisms can be developed as biomarkers for LQTS. Haplotype frequencies and linkage disequilibrium analysis revealed a significant association between the three biomarkers. Compound heterozygosity of the polymorphisms influence downstream signalling and *KCNQ1-KCNE1* interactions.

CONSENT

Informed written consent was obtained from the probands and their family members.

ETHICS COMMITTEE APPROVAL

The study has been approved by the Institutional Ethics Committee, Dept. of Genetics, Osmania University, INDIA.

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COMPETING INTEREST

There is no conflict of interest within the authors.

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